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STEROIDAL ALKALOID TERATOGENS: MOLECULAR PROBES FOR INVESTIGATION OF CRANIOFACIAL MALFORMATIONS

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ABSTRACT

Holoprosencephaly, a malformation sequence that results from impaired midline cleavage of the embryonic forebrain, is expressed as a spectrum of craniofacial anomalies of which cyclopia is the most severe. The *Veratrum* alkaloids are the most prominent of the teratogenic agents known to induce holoprosencephaly in mammals. Jervine and 11-deoxojervine (cyclopamine) are potent steroidal alkaloid teratogens from *Veratrum californicum* that are responsible for inducing cyclopic malformations in sheep.

Extensive structure-terata investigations of jervanes, solanidanes, and spirosolanes have shown that teratogenicity induced upon oral administration of all three structural types is significantly higher if the C-5, C-6 bond is unsaturated. Research in progress on the pathogenesis

of holoprosencephalic malformations in both hamsters and humans offers the potential to provide information on the receptors involved in the expressions of these craniofacial syndromes. A clearer understanding of steroidal alkaloid-induced teratogenesis will emerge when appropriate receptor sites are revealed with which teratogenic alkaloids of slightly different structure can interact.

INTRODUCTION

Cyclopia has fascinated humankind since ancient times. In Greek mythology, the Cyclopes were a race of one-eyed giants who lived as shepherds on the coast of Sicily and were described in detail in Homers' *The Odyssey* (Mandelbaum, 1990). Descriptions of cyclopia are relatively common in the writings of natural philosophers of the 16th and 17th centuries and often were rather inaccurate, fanciful and imaginative (Cohen and Sulik, 1992). Cyclopia has continued as a subject of artistic expression into the 19th and 20th centuries as illustrated by Odilon Redon's lithograph of a smiling cyclops in 1863 and the surrealist artist Rene Magritte's 1963 oil painting entitled *The Difficult Crossing* (Cohen and Sulik, 1992).

Holoprosencephaly is a malformation sequence in which impaired midline cleavage of the embryonic forebrain is the defining feature (Cohen and Sulik, 1992). The prosencephalon fails to cleave sagittally into cerebral hemispheres, transversely into telencephalon and diencephalon, and horizontally into olfactory and optic bulbs. Various gradations of facial dysmorphism are commonly associated with holoprosencephaly in humans (Figure 1). For example, the spectrum of severe anomalies associated with holoprosencephaly includes (A) cyclopia with a single median eye and lack of proboscis, (B) similar to A with various degrees of doubling of the ocular structures, (C) similar to B with formation of an abnormal proboscis, (D) closely-spaced eyes (ocular hypotelorism) with an abnormal proboscis, (E) cebocephaly having a proboscis with a single nostril and (F) median cleft lip and closely-spaced eyes (Nishimura and Okamoto, 1976).

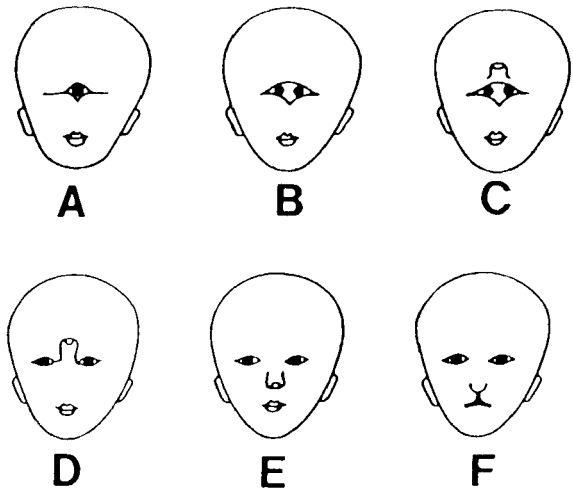


Figure 1. Holoprosencephaly in humans. Nishimura, H. and Okamoto, N. (1976). *Sequential Atlas of Human Congenital Malformations: Observations of Embryos, Fetuses, and Newborns.* University Park Press, Baltimore.

Although human holoprosencephaly may be caused by diabetes (Muenke, 1994) and the disease may be induced in mice by ethyl alcohol (Cohen and Sulik, 1992), the *Veratrum* alkaloids are the most prominent of the teratogenic agents known to induce holoprosencephaly in animals (Keeler, 1984, 1986). During the early part of the 20th century, epidemics of congenital craniofacial malformations in newborn lambs were common on sheep ranches in several National Forests of the western United States. By the 1950s, some ranchers in parts of Idaho experienced an incidence of congenitally malformed lambs of up to 25% of live births. The most common syndrome was called "monkey-face" lamb disease by Basque sheepherders. This double-globe cyclopia was expressed as two closely-spaced corneas in a single distorted, often dumbbell-shaped, sclera. Many afflicted animals were truly cyclopic with a single median eye (Figure 2); in severe examples of the disease, the eyes and nose were absent and the cerebrum rudimentary (Binns et al., 1959, 1960, 1962). Related malformations included; anophthalmia (the complete

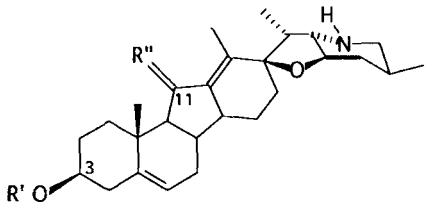
Figure 2. Cyclopic lamb from fourteenth day gestational period maternal dosing of cyclopamine.

absence of ocular tissue), cebocephaly (a misshapen nasal passage), and mandibular hyperplasia (pronounced curvature of the lower jaw) accompanied by maxillary or premaxillary hypoplasia. "Monkey-face" lambs carried to full term or beyond (prolonged gestation) usually are alive at the time of delivery but die soon thereafter because of the severity of their anomalies. Single or twin "monkey-face" lambs frequently grow to enormous size before delivery by Caesarian-section. Prior to the late 1950s, the congenital malformations were thought to be of genetic origin because a "monkey-face" lamb often was born as a twin to a normal lamb. However, a genetic basis for the disease was ruled out by breeding experiments (Binns et al., 1959). The incidence of the disease appeared to correlate both with the range area grazed by afflicted ewes and certain plant species present in those areas. About 1960, field study investigations and experimental feedings had begun which established that the malformations arose when ewes fed on the range plant *Veratrum californicum* during the second and third weeks after conception (Binns et al., 1963). *V. californicum* (Figure 3), commonly known as false hellebore or corn lily, thrives at higher elevations in moist geographically limited areas in mountains of the Pacific Coast and northern

Figure 3. *Veratrum californicum*

Rocky Mountain states. Because of grazing practices, the pregnant ewes were ingesting the teratogenic *Veratrum* plants during the period that they were susceptible to terata induction (Binns et al., 1965). A practical solution to the problem resulted when researchers suggested that ranchers keep their pregnant ewes away from areas containing *V. californicum* until the ewes had passed the fourteenth day of gestation, which was experimentally established as the precise insult day (Binns et al., 1965; Keeler and Binns, 1968). Consequently, the incidence of "monkey-face" lamb disease became virtually non-existent (Keeler, 1978a).

The culmination of the extensive research of Keeler and his colleagues at the Poisonous Plant Research Laboratory was the isolation of three jerveratrum alkaloids from *V. californicum*: jervine, cyclopamine (11-deoxojervine), and its 3-glucosyl derivative (cycloposine) (Figure 4). Each of these alkaloids induced "monkey-face" lamb disease when orally administered to ewes on the fourteenth day of gestation, which is during the primitive streak/neural plate stage of embryonic development in sheep (Keeler and Binns, 1968).



	R'	R"
JERVINE	H	O
CYCLOPAMINE	H	H_2
CYCLOPOSINE	D-Glc	H_2

Figure 4. Teratogenic alkaloids from *Veratrum californicum*.

Experiments in other animals have shown that the activity of the jerveratrum alkaloids was not specific for sheep because cattle and goats also produced malformed offspring upon ingestion of *V. californicum* (Binns et al., 1972). However, as with sheep, cattle and goats possess rumen microorganisms that might alter the jerveratrum alkaloid teratogens by metabolism. Experiments in monogastric mammals revealed that rabbits, mice, rats and hamsters also were susceptible to terata-induction by cyclopamine, presumably without conversion of the cyclopamine to other compounds (Keeler, 1978b). Several subsequent studies in animals and *in vitro* have indicated that jerveratrum alkaloid teratogens exert their effects directly on the embryo (Bryden and Keeler, 1973; Bryden et al., 1973; Omnell et al., 1990). Rabbits were susceptible to cyclopamine administered on the seventh day of gestation, exhibiting double-globe cyclopia and cebocephaly either with a single- or closely-spaced double nostril (Keeler, 1971). Hamsters ultimately became the assay model of choice for plant alkaloid teratogen research due to the relatively small dose of pure alkaloid required (approximately 20-30mg/animal), their short gestation period (16 days), the high incidence of terata induced, and their relative ease of handling (Keeler, 1975).

Three major types of craniofacial malformations are induced in hamsters upon oral administration of plant alkaloids during the primitive streak/neural plate phase, which is on the eighth day of gestation (Gaffield et al., 1992). These primary malformations include: exencephaly (fully-exposed brain), which appears to be a developmental defect similar to anencephaly in humans; encephalocele (partially exposed brain), which is a herniation of the brain that may appear as a protrusion of meningeal or skin-covered brain tissue; and cebocephaly, which is expressed as a misshapen nasal chamber with an absent or incomplete nasal septum and is part of the holoprosencephaly spectrum of forebrain malformations. Often, closely-spaced eyes with a single-nostril nose are associated with cebocephaly. A tabulation of all craniofacial malformations induced in hamsters by oral administration of steroidal alkaloids has been recorded (Gaffield and Keeler, 1996a).

STRUCTURE - TERATA CORRELATIONS

Each of the teratogenic expressions induced by *Veratrum* in sheep, which include not only craniofacial, but also limb, palate, and tracheal malformations that are induced at later gestational periods (Keeler, 1988), has related counterparts in humans (Warkany, 1971). The steroidal alkaloid *Veratrum* teratogens are somewhat related in structure to the spirosolane and solanidane alkaloids from the *Solanum* and other genera. Furthermore, *Solanum* alkaloid-containing species often are used as human foodstuffs (Facciola, 1990). For these reasons, numerous investigations of steroidal alkaloid-induced teratogenesis have been conducted during the past two decades. The most prominent study involved the controversial hypothesis of Renwick (1972) that suggested a correlation between human congenital malformations such as anencephaly and spina bifida with the consumption of blighted potatoes. Although the hypothesis was essentially disproved within three years (Nevin and Merrett, 1975), Renwick championed the incorporation of vitamin supplements containing folate and ascorbate (Pregnavite Forte F) into maternal diets

for the prevention of anencephaly/spina bifida (Renwick, 1982a, 1982b). Persuasive evidence supporting a protective effect of folate against neural tube defects has been reported (Beresford, 1994). Renwick's research was a forerunner of worldwide efforts that have resulted in the first major change in food fortification in over half a century; the U. S. Food and Drug Administration has implemented a program that will increase dietary folate intake by fourfold enrichment of flour beginning in 1996 (Hine, 1996).

Experimental studies in hamsters of terata induced upon oral administration of jervanes, solanidanes and spirosolanes have shown that all three structural types were significantly less teratogenic upon saturation of the C-5, C-6 linkage (Figure 5) (Gaffield and Keeler, 1993). Ninety-two percent of the fetuses obtained from animals administered jervine were malformed whereas terata occurred in only 14% of fetuses derived from tetrahydrojervine-dosed hamsters. Similarly, the percentage of malformed fetuses derived from solanidine-treated hamsters declined from 24 to 3 percent and the percentage of malformed fetuses from solasodine-treated animals declined from 29 to 6 percent upon saturation of the C-5, C-6 olefinic bond (Gaffield and Keeler, 1993). Clearly, hamster teratogenicity induced upon oral administration of jervanes, solanidanes, and spirosolanes appears to correlate closely with the presence or absence of C-5, C-6 unsaturation in these three classes of steroidal alkaloids. The effect of molecular configuration at stereocenters near the amino group plays a lesser, yet significant, role in the teratogenicity both of solanidanes and spirosolanes (Gaffield and Keeler, 1996a, 1996b). The relationship of molecular configuration to the teratogenicity of jervanes remains to be established. Energy-minimized structures of jervanes, solanidanes, and spirosolanes may provide valuable insight into the teratogenic importance of the availability of negative charge relative to the steroidal framework.

On the basis of the extensive structure-terata information available, a Table of relative teratogenic potencies of steroidal alkaloids in hamster litters was proposed based upon

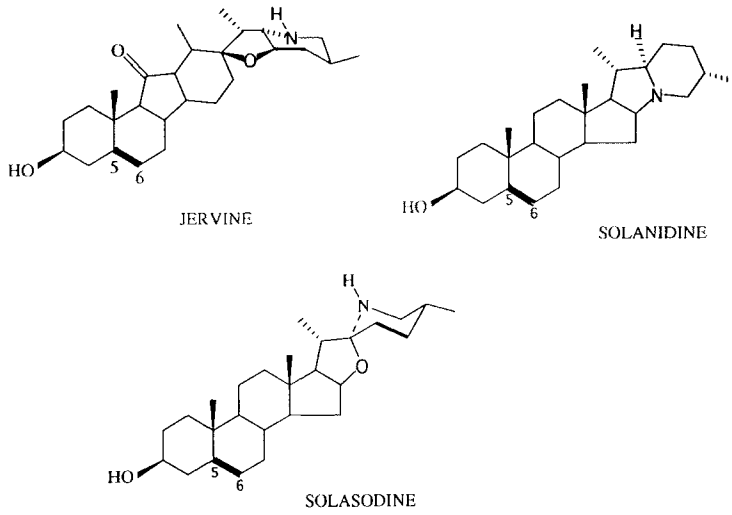


Figure 5. Teratogenic implications of C-5,C-6 unsaturation in steroidal alkaloids.

extrapolation both of recent data and older literature results to equivalent oral dosage (Table 1) (Gaffield and Keeler, 1996a). The potencies range from the most teratogenic jerveratrum alkaloid, jervine, to the nonteratogenic spirosolane, tomatidine. Based upon malformed litters, this tabulation is proposed only as an approximate correlation and as a qualitative rather than a quantitative assessment. Teratogenicity data incorporated into the relative teratogenic potencies were obtained from differently controlled animal experiments that were conducted at two research laboratories using hamsters from the same supplier but whose relative teratogenic susceptibilities may have varied. However, the relative teratogenic potencies shown in Table 1 may serve as a guide for comparison of structure-terata relations and for estimating the mammalian teratogenicity of newly-discovered steroidal alkaloid glycosides and their aglycones. Teratogenic potencies of jervanes and solanidanes are appreciably higher than those of spirosolanes while the potency of jervanes is generally greater than that of solanidanes.

Table 1. Relative Teratogenic Potencies of Steroidal Alkaloids.

Alkaloid	Relative Teratogenic Potency
JERVINE	100
DIHYDROJERVINE	65
S,R-SOLANIDANES	50
CYCLOPAMINE	47
CHACONINE	43
TETRAHYDROJERVINE	40
R,S-SOLANIDINE	32
SOLANIDINE-N-OXIDE	32
SOLANINE	32
DIHYDROSOLANIDINE	9
MULDAMINE	9
SOLASODINE	6
DIHYDROSOLASODINE	4
TOMATINE	1
TOMATIDINE	0

W. Gaffield and R.F. Keeler, *J. Nat. Toxins* **5:** 25-38(1996). Structures are shown in the Figures and the Appendix.

These terata-inducing correlations are emphasized in Figure 6 where values of the relative teratogenic potency of individual alkaloids, abstracted from Table 1, are listed adjacent to their structural type. For example, values for jervine (100), dihydrojervine (65), cyclopamine (47), and tetrahydrojervine (40) are shown adjacent to the jervine structure that represents jervanes in general. The variance in values for the jervanes, solanidanes, and spirosolanes primarily reflects

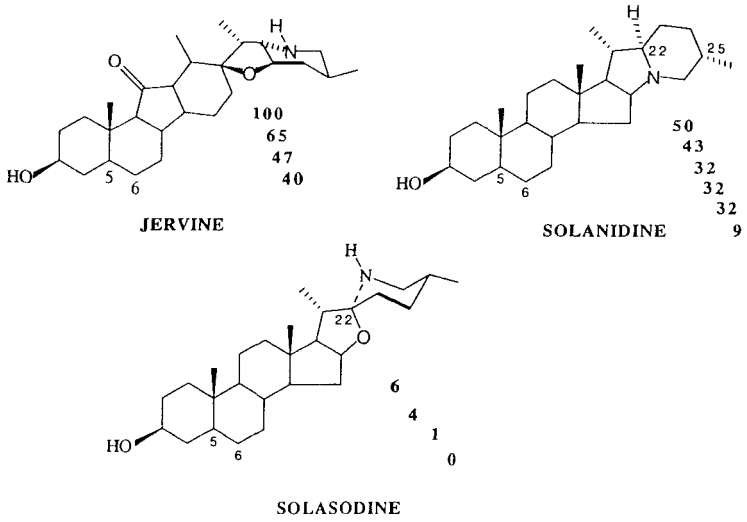


Figure 6. Correlation of relative teratogenic potency (from Table 1) with steroidal alkaloid structure. For example, values for solasodine (6), dihydrosolasodine (4), tomatine (1), and tomatidine (0) are shown adjacent to the solasodine structure that represents spirosolanes in general.

the level of saturation at C-5, C-6; e.g., 100 vs 40 for jervine, 32 vs 9 for solanidine, and 6 vs 4 for solasodine (cf, Table 1). However, opposite configurations at C-22 yield relative teratogenic potencies of 50 vs 32 for solanidine and 4 vs zero for spirosolanes. Only solanidanes and the weakly teratogenic spirosolanes occur in human foodstuffs and the levels of solanidanes in potatoes is closely monitored and regulated (Maga 1994; Hopkins, 1995). Presumably the jerveratrum alkaloids pose a teratogenic hazard only to range animals, because they are not known to occur in plants used for human food (Roitman and Panter, 1995).

BIOLOGICAL IMPLICATIONS AND FUTURE RESEARCH

The holoprosencephaly-craniofacial malformation syndrome is a developmental field defect that occurs in roughly 1 of 16000 live births (Roach et al, 1975), although it is considerably

more common in embryogenesis, occurring in approximately 1 of 250 spontaneous abortions (Matsunaga and Shiota, 1977). In addition to the various defects of the craniofacial malformation complex (cf, Figure 1), several syndromes are believed to be associated with holoprosencephaly. Included among these are Martin syndrome, the CHARGE association, and the Pallister-Hall syndrome (Muenke, 1994). One hypothesis for the pathogenesis involves a primary insult to the prechordal mesenchyme during gastrulation, disturbing complex interactions between the developing neuroepithelium and cranial mesenchyme. The hamster model is particularly useful for studying the pathogenesis of holoprosencephalic craniofacial malformations because in this species the *Veratrum* alkaloid-induced facial anomalies may often be isolated from holoprosencephalic malformations of the brain. In a preliminary report of research from Children's Hospital in Seattle, Siebert and his colleagues have followed the expression of two transcription factors that are important in embryogenesis in cyclopamine-treated hamsters (Coventry et al. 1996a, 1996b). One of these, HNF-3β, is a transcription factor expressed in the gastrulating embryo that is essential for node, notochord and floor plate formation and for dorsal-ventral patterning of the neural tube. HNF-3β is decreased transiently along the dorsal midline following cyclopamine administration whereas no changes in expression were observed for Hox-b-5, a homeobox gene expressed in the neural tube (Coventry et al., 1996b). Thus, Siebert and his colleagues have proposed that HNF-3β may be important in the specification of rostral neural crest (Coventry et al., 1996b).

Because of the interest in obtaining a better understanding of normal development of the human brain and face and, ultimately, elucidation of the basic genetic defects which program the abnormal formation expressed in holoprosencephaly, molecular genetic approaches that should lead to the positional cloning of genes involved in holoprosencephaly are being actively pursued (Winter, 1996). In particular, research in progress at Children's Hospital in Philadelphia and at the University of Pennsylvania School of Medicine led by Muenke has focused attention on

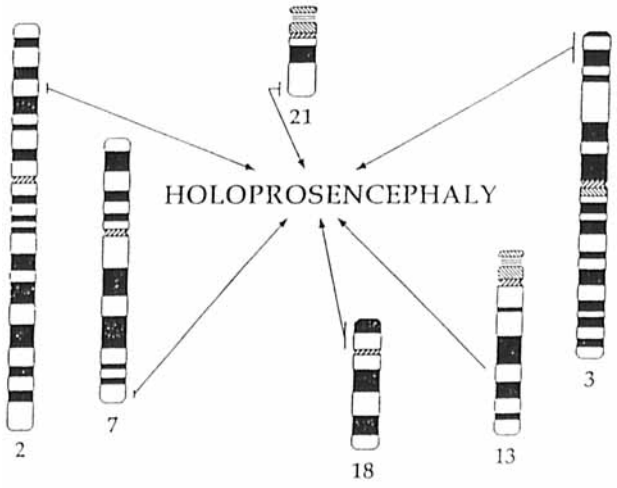
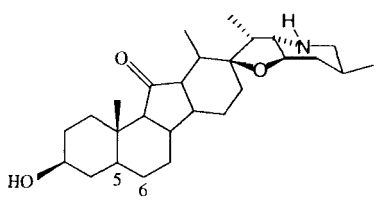


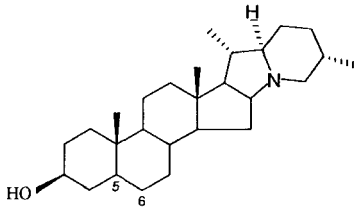
Figure 7. Chromosomal regions involved in holoprosencephaly. Muenke, M. (1994). Holoprosencephaly as a genetic model for normal craniofacial development. *Semin. Dev. Biol.* **5**, 293-301.

several human chromosomal regions involved in holoprosencephaly (Figure 7) that may host possible candidates underlying the genetic causes of the expression (Muenke, 1994). These chromosomal regions, four of which have been designated as 21q22.3, 2p21, 7q36, and 18p, are believed to contain genes that are crucial for normal forebrain and midface development (Frézal and Schinzel, 1991). In practice, these molecular genetic studies should allow carrier detection and prenatal diagnosis in selected families with autosomal dominant holoprosencephaly. Although at present none of these genes has been identified, positional cloning should permit structural clarification of the holoprosencephalic genes that might aid in the identification of related genes which are involved in abnormal midline development of the forebrain and midface. Analysis of the regulation, interaction and physiological role of the holoprosencephalic genes may elucidate mechanisms underlying both normal and abnormal craniofacial development.



JERVANES

BRAIN AND NASAL DEFECTS



SOLANIDANES

BRAIN DEFECTS

Figure 8. Correlation of steroidal alkaloid structure with pattern of craniofacial malformations.

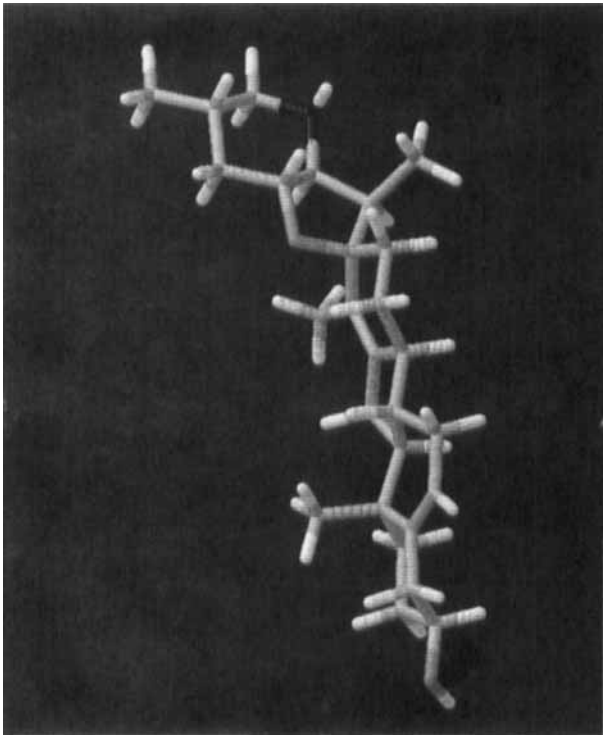


Figure 9. Energy-minimized model of cyclopamine.

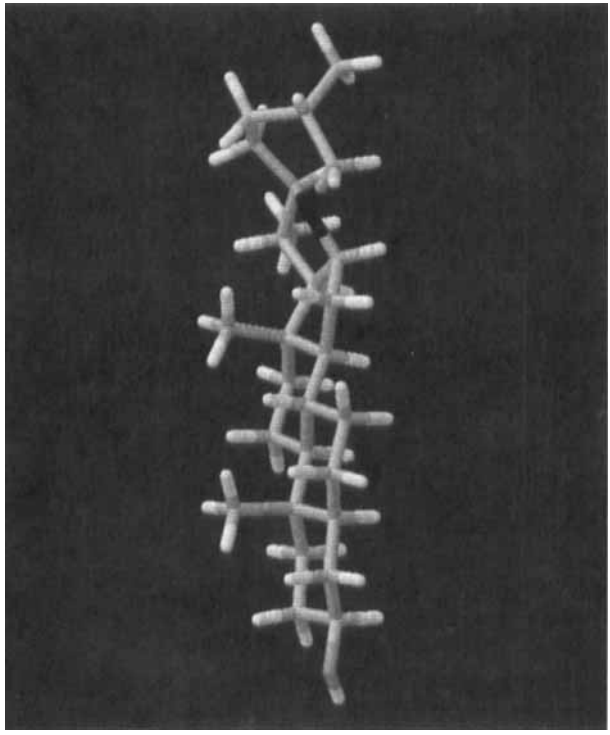


Figure 10. Energy-minimized model of 22(*R*)-solanidine.

The pattern of terata expression in experimental animals has been suggested as a guide to whether or not congenital malformations are induced by the same mechanism (Keeler, 1984, 1986). Thus, jervine and cyclopamine induce both brain (exencephaly and encephalocele) and nasal or forebrain (cebocephaly, cleft lip, and cleft palate) malformations whereas naturally-occurring solanidanes and spirosolanes induce only brain defects (Figure 8) (Gaffield and Keeler, 1996a). Molecular modeling offers the potential to delineate the subtle structural differences between these closely-related alkaloids that in one of two structures permit induction of nasal or forebrain malformations which are part of the holoprosencephalic spectrum. Energy-minimized models of cyclopamine (Figure 9) and solanidine (Figure 10) clearly depict the structural

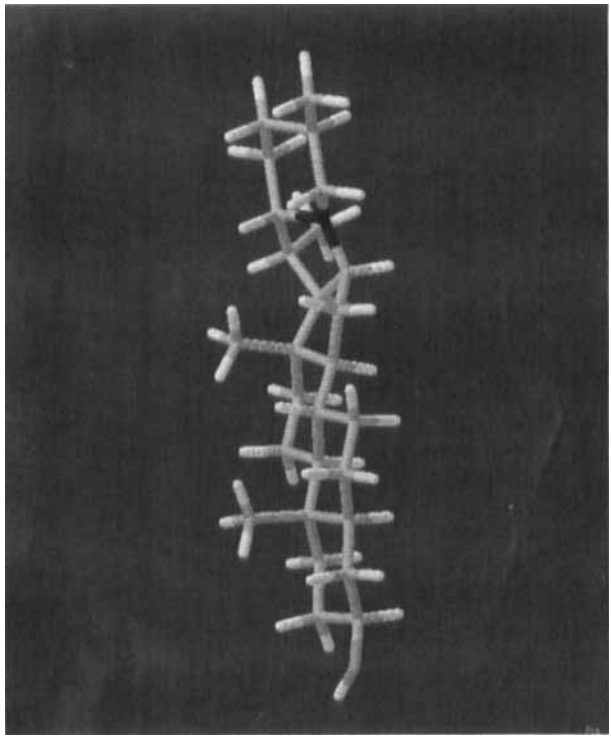


Figure 11. Energy-minimized model of 22(*S*)-solanidine.

differences between these two alkaloids that induce different malformation patterns, particularly in rings E and F (cf, Appendix) where the spirofuranopiperidine moiety of cyclopamine assumes an orientation perpendicular to the planar steroidal framework in contrast to the extended solanidine structure. Reduction of jervine's double bonds at C-12, C-13 and C-5, C-6 not only greatly lowers the teratogenicity of the alkaloid but also alters the pattern of malformations. Tetrahydrojervine induces only brain malformations which may imply that the unsaturation at C-5, C-6 of the steroid ring system contributes significantly to complex stability, although it is uncertain whether this effect is a result of favorable interactions of the polarizable double bond with a receptor or due to differences in the steric fit inside a receptor cavity.

An even subtler structural difference between alkaloids that may or may not induce nasal or forebrain malformations might be represented by solanidanes that are epimeric at C-22. 22-*S*-Solanidine (Figure 11) induced a significant amount of cleft lip in hamsters (Brown and Keeler, 1978), whereas the epimeric naturally-occurring 22-*R*-solanidine (Figure 10) did not (Gaffield and Keeler, 1993). A caveat stresses that because these epimeric solanidanes were evaluated for teratogenicity in different experiments, comparison of terata patterns may not be valid due to differing terata susceptibility of the animals, even though of the same strain and from the same supplier, or due to a variation in either absorption or clearance rates that could provide a shift in insult timing (Keeler, 1984, 1986). However, a structural difference between these alkaloids involves the pyramidal tertiary nitrogen atom which, with its unshared electron pair, is projected below the steroidal plane in the 22-*S*-solanidane (Figure 11) and above the steroidal plane in the 22-*R*-isomer (Figure 10).

These studies will be greatly facilitated when molecular biological research, as mentioned above, reveals an appropriate receptor site at which the teratogenic alkaloids of slightly different structure can interact. A clearer understanding of the role of steroidal alkaloid teratogens effect on, or inhibition of, specific biochemical mechanisms *in vitro* that are involved in mammalian teratogenesis may then emerge.

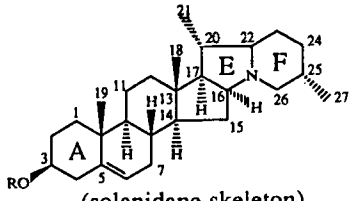
ACKNOWLEDGMENT

The authors express appreciation to Dr. Douglas Henry, MDL Information Systems Inc., San Leandro, CA for providing the structures shown in Figures 9-11.

APPENDIX

Structures for jervine and cyclopamine are shown in Figure 4; dihydrojervine has the 12, 13-bond (adjacent to the 11-carbonyl) saturated and tetrahydrojervine has both the 12, 13-and 5,

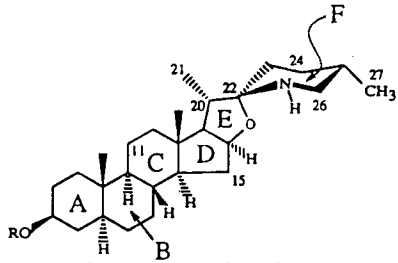
6-bonds saturated. Dihydrosolanidine is depicted in Figure 6 as the alkaloid structural type labelled solanidine. 22*R*, 25*S*-Solanidine is shown below as the solanidane skeleton with R=H. In solanidine N-oxide, the electron pair on the nitrogen atom of solanidine is replaced by an oxygen atom. 22*S*, 25*R*-Solanidine is shown in Figure 11. Dihydrosolasodine is depicted in Figure 6 as the alkaloid structural type labelled solasodine. Solasodine has this structure with unsaturation at C-5, C-6. Structures for α-solanine, α-chaconine, tomatine, tomatidine, and muldamine are illustrated below.



(solanidane skeleton)

α–solanine R = β–D–glucose (1→3) β–D–galactose (1→3)
α–L–rhamnose (1→2) β–D–galactose

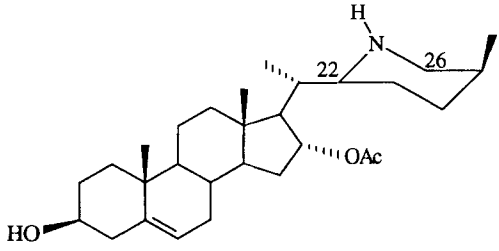
α–chaconine R = α–L–rhamnose (1→4) β–D–glucose (1→3)
α–L–rhamnose (1→2) β–D–glucose



(spirosolane skeleton)

tomatine R = xylose, glucose (1→2) glucose (1→4) galactose

tomatidine R = H



muldamine

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